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Asparagine Residue in the *rho* Gene Product Is the Modification Site for Botulinum ADP-ribosyltransferase*

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We reported previously that the ADP-ribosyltransferase in C_1 and D botulinum toxins specifically catalyzes ADP-ribosylation of an M_r 22,000 guanine nucleotide-binding protein and that this substrate named G_b (b = botulinum) has an amino acid sequence homologous to that deduced from the *rho* gene (Narumiya, S., Sekine, A., and Fujiwara, M. (1988) *J. Biol. Chem.* 263, 17255–17257). In this study we have determined the amino acid sequence at its ADP-ribosylation site. Purified substrate was [^{32}P]ADP-ribosylated by C_1 botulinum toxin and digested with trypsin. The radioactive peptides were isolated by reversed-phase high performance liquid chromatography and digested further either with protease V8, with proteases V8 and thermolysin, or with proline endopeptidase and thermolysin. By this procedure three radioactive peptides were obtained, and their amino acid sequences were *X*-Tyr-Val-Ala-Asp-Ile-Glu, *X*-Tyr, and Val-Phe-Glu-*X*-Tyr in which no amino acid peak was found in *X*. During the sequencing the radioactivity quantitatively adhered to the sequencing filter and was not eluted with either of the identified amino acid residues. Analysis of the protein without the ADP-ribosylation yielded the corresponding sequence as Thr-Val-Phe-Glu-Asn-Tyr which corresponds to Thr^{37}–Tyr^{42} in the amino acid sequence deduced from the *Aplysia rho* gene. These results strongly suggest that the asparagine residue is the ADP-ribosylation site in the *rho* gene product. This ADP-ribose protein bond was stable in 0.5 M hydroxylamine at pH 7.5 at 37 °C for at least 5 h. The ADP-ribosylation of this protein affected neither its GTPase- nor its [^{35}S]guanosine 5′-*O*-thiotriphosphate-binding activity.

In 1987 we first discovered that two types of botulinum neurotoxin, C_1 and D, contain an ADP-ribosyltransferase which specifically ADP-ribosylates an M_r 21,000–22,000 cellular protein (1, 2). Our finding was confirmed later by several other groups (3, 4). Concurrent with our reports, Aktories *et al.* (5) found another ADP-ribosyltransferase, C_3, in culture filtrate of type C strain of *Clostridium botulinum* which appears to ADP-ribosylate the same M_r 21,000 protein. The ADP-ribosylation of this unknown substrate was stimulated by the two known ligands of G-protein, guanine nucleotides and magnesium ion, suggesting that it is a member of G-proteins[1] (1–6). However, the identity of this protein was not clarified until we had purified it from the cytosol of bovine adrenal gland and identified as an M_r 22,000 G-protein (7). On the basis of these results we have named it G_b (b = botulinum). We have further clarified the identity of G_b by determining its partial amino acid sequences. Homology analysis of these sequences revealed that they are highly homologous to that deduced from *Aplysia rho* gene and suggested that G_b is a putative *rho* gene product (8).

The purified protein was maximally ADP-ribosylated to the extent of about 0.7 mol of ADP-ribose/mol of protein, suggesting that there is only one ADP-ribosylation site in a molecule. However, the amino acid at the ADP-ribose acceptor site has not been identified yet. Other bacterial ADP-ribosyltransferases catalyze the modification of the specific amino acid residues (9). For example, diphtheria toxin ADP-ribosylates a modified histidine residue, diphthamide, in elongation factor 2 (10). Pertussis toxin transfers the ADP-ribose to a cysteine residue in transducin as well as G_i/G_o (11). Cholera toxin catalyzes the ADP-ribosylation of an arginine in G_s and transducin (12). The amino acid acceptor in this protein was initially suggested to be an arginine residue since several guanidine-containing compounds including L-arginine inhibited ADP-ribosylation by C_1 toxin (1, 2). However, unlike cholera toxin, the botulinum toxin did not transfer the ADP-ribose moiety to L-arginine itself (1). It was also questioned recently by Aktories *et al.* (13) who found that this ADP-ribose protein bond was different from an ADP-ribose arginine linkage in sensitivity to hydroxylamine or alkali. We now present several lines of evidence to suggest that an asparagine residue is the ADP-ribosylation site of G_b, the *rho* gene product.

EXPERIMENTAL PROCEDURES

Materials—G_b was purified from the cytosol of bovine adrenal glands as described previously (7). C_1 botulinum toxin (L-form) was a kind gift of Professor G. Sakaguchi of Osaka Prefectural University. [α-^{32}P]NAD (34 Ci/mmol), [^{35}S]GTPγS (1,350 Ci/mmol), [α-^{32}P]GTP (33 Ci/mmol), and [γ-^{32}P]GTP (30 Ci/mmol) were obtained from Du Pont-New England Nuclear. Trypsin from bovine pancreas type III (10,200 benzoyl-L-arginine ethyl ester units/mg of protein) was obtained from Sigma. Protease V8 from *Staphylococcus aureus* (550 units/mg of material) was obtained from ICN Immuno-Biologicals. Proline endopeptidase from *Flavobacterium meningosep-*

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[1] The abbreviations used are: G-protein, guanine nucleotide-binding protein; G_b, a guanine nucleotide-binding protein which is specifically ADP-ribosylated by *C. botulinum* ADP-ribosyltransferase and homologous to a putative *rho* gene product; GTPγS, guanosine 5′-*O*-thiotriphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; GAP, GTPase-activating protein.

ticum (35 units/mg of protein) was obtained from Seikagaku Kogyo, Tokyo, Japan. Thermolysin from *Bacillus thermoproteolyticus* (8,670 units/mg of protein), trifluoroacetic acid (HPLC grade), and hydroxylamine hydrochloride were obtained from Nacalai Tesque, Kyoto, Japan. An Aquapore RP-300 column (2.1 mm, inner diameter, × 30 mm) and a Spheri 5RP-18 column (2.1 mm, inner diameter, × 30 mm) were obtained from Applied Biosystems, Inc. The phenylthiohydantoin derivative standard kit was purchased from Pierce Chemical Co. Norit A was obtained from Wako Pure Chemical, Osaka, Japan. All other chemicals used were of reagent grade.

Preparation of [^{32}P]ADP-ribosylated G_b—Partially purified G_b after the TSK gel column chromatography (7) was incubated with 50 μM [α-^{32}P]NAD (1.25 μmol/pmol of G_b, 1000 cpm/pmol) and C_1 toxin (0.125 μg/pmol of G_b) in 100 mM Tris·HCl, pH 8.0, containing 10 mM thymidine, 10 mM dithiothreitol, 10 mM nicotinamide, and 5 mM $MgCl_2$ for 3 h at 30 °C. After the incubation, the mixture was concentrated at 4 °C to 500 μl by the use of a Centricon 10 (Amicon), and the concentrate was subjected to Mono Q fast protein liquid chromatography as described previously (7). [^{32}P]ADP-ribosylated G_b was eluted later in the gradient than unmodified G_b and could therefore be freed of this and other contaminating proteins. The G_b amount was determined by measuring the ^{32}P radioactivity incorporated into the M_r 22,000 protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Protease Digestion, Isolation of Radioactive Peptides, and Amino Acid Sequencing—We digested [^{32}P]ADP-ribosylated and unmodified G_b with combinations of various proteases as described in the following four experiments. Radioactive peptides recovered were quantified by measuring the ^{32}P radioactivity. In experiment 1, [^{32}P]ADP-ribosylated G_b (73 μg of protein, 3.3 nmol) was incubated with 33 pmol of trypsin in 700 μl of Mono Q buffer (7) containing 10 mM $CaCl_2$ at 30 °C. At 9 h of incubation, another 33 pmol of trypsin was added, and incubation was carried out for another 9 h. The mixture was then acidified by the addition of trifluoroacetic acid to a final concentration of 0.1% and subjected to reversed-phase HPLC on an Aquapore RP-300 column connected to a Beckman HPLC System Gold. HPLC was performed at a flow rate of 0.2 ml/min with water containing 0.1% trifluoroacetic acid (solvent A) for the first 15 min and then with a linear gradient between 0 and 54% acetonitrile in solvent A for 45 min. Fractions were collected every 1 min. Elution of peptides was monitored by absorbance at 210 nm, and that of the radioactivity was monitored by measuring the radioactivity in 10-μl aliquots of each fraction. As shown in Fig. 1, the tryptic digestion produced four radioactive peaks in more than 10 peptide peaks. The radioactive peptides in the four peaks (I, 0.32 nmol; II, 0.49; III, 0.96; IV, 0.84) were collected and further digested with protease V8 (11, 16, 32, and 28 pmol for I, II, III, and IV, respectively) in 100 mM potassium phosphate, pH 7.8, at 30 °C. At 8 h of incubation, the same amounts of protease V8 were added to each mixture, and incubation was carried out for another 8 h. The mixture was then acidified and subjected to the reversed-phase HPLC as described above. Elution of protease V8 fragments was performed with solvent A for the first 15 min and then with a linear gradient between 0 and 60% acetonitrile in solvent A for 60 min. A single radioactive peptide was eluted at a retention time of 30 min from the protease V8 digestions of all of the tryptic fragments I–IV; I, 0.27 nmol; II, 0.13; III, 0.95; IV, 0.34. Amino acid sequences of these peptides were determined with an automated pulse-liquid phase protein sequenator, Applied Biosystems Inc. model 477A, equipped with an on-line phenylthiohydantoin derivative analyzer, Applied Biosystems Inc. model 120A, as described previously (8).

In experiment 2, a different batch of purified ADP-ribosylated G_b (79 μg of protein, 3.6 nmol) was digested with trypsin and protease V8 as described in experiment 1. The protease V8 digests of each of four tryptic fragments (I–IV) underwent the HPLC separation as described above, and the radioactive peptides eluted at a retention time of 30 min from the four samples were combined (3.4 nmol). A part of the combined fraction (0.69 nmol) was incubated with 7 pmol of thermolysin twice every 3 h in 20 mM Tris·HCl, pH 7.8, containing 10 mM $CaCl_2$ at 30 °C. The digests were acidified and subjected to reversed-phase HPLC as described above. The radioactive product (0.25 nmol) which passed through an Aquapore RP-300 column was applied to a Spheri 5RP-18 column. Elution was performed as described for the protease V8 digests. A single radioactive peptide (0.09 nmol) was eluted at a retention time of 24 min and subjected to amino acid sequencing.

In experiment 3, another batch of purified ADP-ribosylated G_b (55 μg of protein, 2.5 nmol) was incubated with trypsin as described in experiment 1. The four radioactive peaks of the tryptic digests were combined (1.9 nmol) and incubated with 2 μg of proline endopeptidase twice every 8 h in 100 mM potassium phosphate, pH 7.0, at 30 °C. The mixture was acidified and subjected to reversed-phase HPLC on an Aquapore RP-300 column as described above. Elution of the fragment peptides was performed with solvent A for the first 15 min and then with a linear gradient between 0 and 50% acetonitrile in solvent A for 45 min. A radioactive peak was eluted at a retention time of 32 min, and this fraction containing 0.95 nmol of radioactive peptides was further digested with thermolysin. The digests were then subjected to the reversed-phase HPLC on a Spheri-5RP-18 column as described in experiment 2. A single radioactive peptide was obtained at a retention time of 48 min (0.36 nmol), and the amino acid sequence of this peptide was determined as described above.

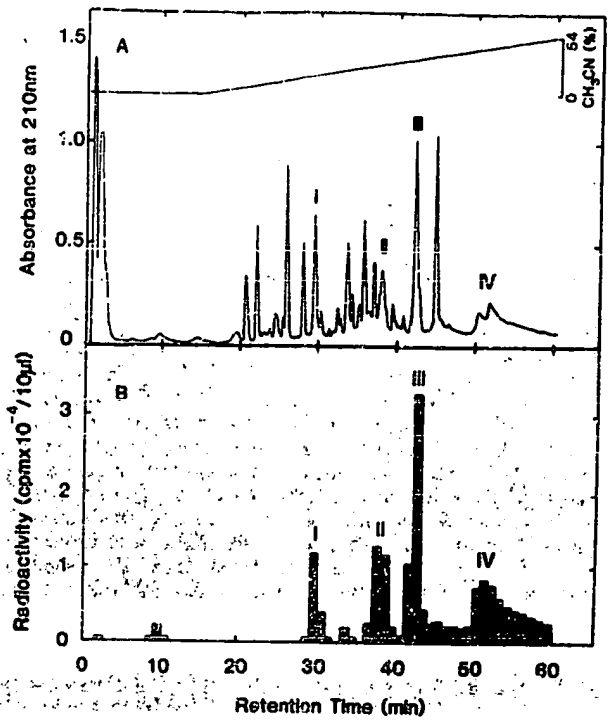

FIG. 1. Reversed-phase HPLC of tryptic digests of [^{32}P]ADP-ribosylated G_b. Purified [^{32}P]ADP-ribosylated G_b was digested with trypsin, and the digestion products were applied to a reversed-phase HPLC as described under "Experimental Procedures." *A*, elution profile of peptides monitored by absorbance at 210 nm; *B*, elution profile of the radioactivity. Radioactive peaks were numbered *I–IV* as shown in this figure.

In experiment 4, unmodified G_b (70 μg of protein, 3.2 nmol) was incubated with 32 pmol of trypsin twice every 9 h. The mixture was then acidified and subjected to the reversed-phase HPLC as described in experiment 1. Peaks from tryptic digestion were further incubated with 1 μg of proline endopeptidase as described in experiment 3. Each mixture was then acidified, and the digestion fragments were separated by the reversed-phase HPLC on an Aquapore RP-300 column as described in experiment 2. Amino acid sequences of isolated peptides were determined with a protein sequenator until the sequence corresponding to those of the [^{32}P]ADP-ribosylated peptides was obtained.

Sensitivity to Hydroxylamine—Sensitivity of the ADP-ribose G_b bond to hydroxylamine was examined as described by Hsia *et al.* (14) for the analyses of ADP-ribose cysteine and ADP-ribose arginine linkages. Purified [^{32}P]ADP-ribosylated G_b (200 pmol, 630 cpm/pmol) was incubated at 37 °C either with freshly prepared 0.5 M hydroxylamine or with 0.5 M NaCl in 50 mM Hepes·NaOH, pH 7.5, in a total volume of 2 ml. At various times, 200-μl aliquots were taken, and the protein was precipitated by the addition of 400 μl of 0.02% sodium deoxycholate and 200 μl of 24% trichloroacetic acid (7). The precipitates were then solubilized in a Laemmli sample buffer and applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 15% gel. The protein bands corresponding to M_r 21,000–22,000 substrate were excised, and the radioactivity was determined on a Packard Minaxi model 4430 liquid scintillation counter.

[^{35}S]GTPγS Binding and GTPase Activities of ADP-ribosylated G_b—Purified G_b (84 pmol) was incubated with 100 μg of C_1 toxin either in the presence or absence of 70 μM NAD in 280 μl of 100 mM Tris·HCl, pH 8.0, containing 10 mM dithiothreitol and 5 mM $MgCl_2$ at 30 °C for 1 h. After the incubation, each mixture was divided into two halves. One half was used for measurement of GTPγS-binding activity, and the other half for that of GTPase activity. GTPγS-binding activity was measured as described previously (7). GTPase activity was measured as described by Brandt *et al.* (15) with some modifications. Briefly, 42 pmol of ADP-ribosylated G_b or unmodified G_b from the first incubation was incubated with 1 μM [γ-^{32}P]GTP (30 Ci/mmol) at 37 °C in 700 μl of 50 mM Hepes·NaOH, pH 8.0, containing 0.5 mM $MgSO_4$, 100 mM NaCl, 0.25 mM 5′-adenylylimidodiphosphate, and 5 mM dithiothreitol. At various times, 100-μl aliquots were taken from each mixture and mixed with 900 μl of ice-cold 0.7 M perchloric acid containing 25 mM KH_2PO_4 and 5% Norit A. Samples were chilled on ice for 30 min and centrifuged at 2500 × *g* for 20 min. The radioactivity in 500 μl of supernatant was determined in a Triton-toluene scintillator.

RESULTS

Amino acid sequences of the radiolabeled peptides obtained from digestions of [^{32}P]ADP-ribosylated G_b are shown in Table I. When [^{32}P]ADP-ribosylated G_b was digested with trypsin, four radioactive peaks were obtained on the reversed-phase HPLC which were numbered I–IV in Fig. 1. When peptides in these peaks were further digested with protease V8, all of them yielded a single radioactive peptide eluted at a retention time of 30 min on the second reversed-phase HPLC. These peptides from the protease V8 digestion showed a common amino acid sequence (Table I, experiment 1). Peptides I–III gave the identical sequence, *X*-Tyr-Val-Ala-Asp-Ile and peptide IV, *X*-Tyr-Val-Ala-Asp-Ile-Glu, in which no amino acid was found in *X*. These results suggest that although the retention times of the four tryptic fragments were different from each other, they derived from the same part of G_b protein and confirmed that there is only one ADP-ribosylation site in a G_b molecule. In order to identify further the location of the ADP-ribosylation site, these peptides were combined and digested with thermolysin in experiment 2. In

TABLE I

Amino acid sequences of the radioactive peptides obtained from [^{32}P]ADP-ribosylated G_b

Protease digestion of [^{32}P]ADP-ribosylated G_b, isolation of fragment peptides, and amino acid sequencing were performed as described under "Experimental Procedures."

Experiment	Protease digestions	No.	Sequence
1	Trypsin and protease V8	I, II, III	*X*-Tyr-Val-Ala-Asp-Ile
		IV	*X*-Tyr-Val-Ala-Asp-Ile-Glu
2	Trypsin, protease V8, and thermolysin		*X*-Tyr
3	Trypsin, proline endopeptidase, and thermolysin		Val-Phe-Glu-*X*-Tyr

TABLE II

Sequence analyses of proteolytic peptides

Sequence analyses of the [^{32}P]ADP-ribosylated peptide from experiment 3 and the corresponding peptide from unmodified protein from experiment 4 were carried out as described under "Experimental Procedures." The yield of each cycle was calculated by comparing the height of each phenylthiohydantoin amino acid peak with that of a standard phenylthiohydantoin derivative.

ADP-ribosylated peptide (I)			Unmodified peptide (II)			Ratio I/II
Cycle	Amino acid	Yield	Cycle	Amino acid	Yield	
		pmol			*pmol*	
			1	Thr	88.2	
1	Val	21.5	2	Val	81.3	0.26
2	Phe	33.7	3	Phe	82.1	0.41
3	Glu	23.9	4	Glu	88.6	0.27
4	ND[a]		5	Asn	88.4	
5	Tyr	9.84	6	Tyr	12.7	0.77

[a] ND, not detected

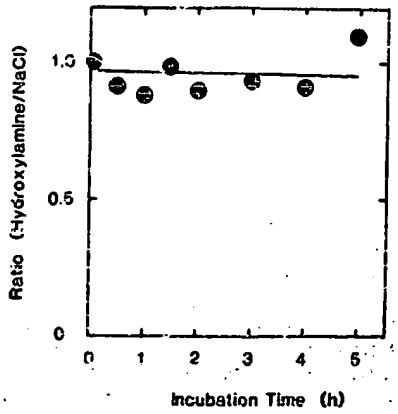

FIG. 2. **Stability of ADP-ribose G_b bond in neutral hydroxylamine.** G_b [^{32}P]ADP-ribosylated by C_1 toxin was incubated at pH 7.5 with 0.5 M hydroxylamine or NaCl at 37 °C for indicated times as described under "Experimental Procedures." The G_b-associated radioactivity was determined in each incubation, and the ratio of the remaining radioactivity in the presence of hydroxylamine and NaCl is shown.

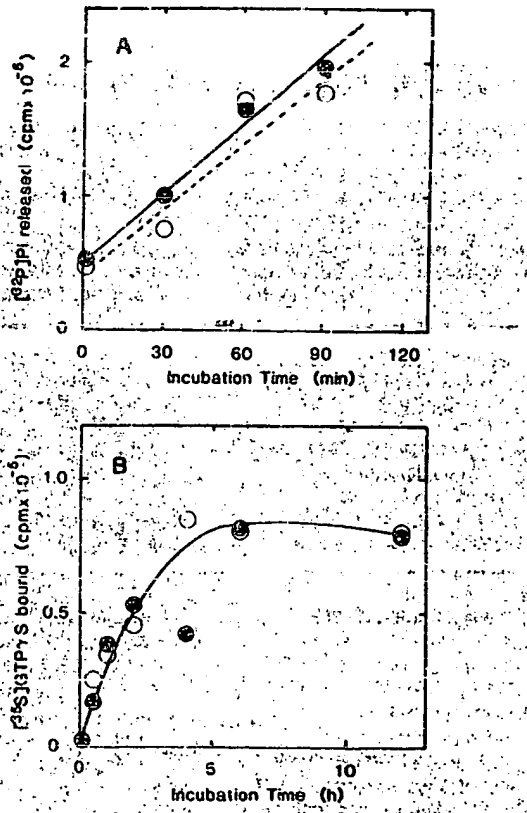

FIG. 3. **GTPase- (*A*) and [^{35}S]GTPγS-binding (*B*) activities in ADP-ribosylated and unmodified G_b.** GTPase- and GTPγS-binding activities were measured in the ADP-ribosylated (●) and unmodified G_b (○) as described under "Experimental Procedures."

this experiment, a single radioactive peptide was obtained and yielded a sequence of *X*-Tyr (Table I, experiment 2), suggesting that the [^{32}P]ADP-ribose was present in the first 2 residues of the above peptides. We further confirmed this finding by obtaining another peptide containing an overlapping sequence. To this we digested the tryptic fragments sequentially with proline endopeptidase and thermolysin in experiment 3. The radioactive peptide isolated by this procedure yielded a sequence of Val-Phe-Glu-*X*-Tyr (Table I, experiment 3). Thus, the overlapping sequence was *X*-Tyr, confirming that the ADP-ribose bound to either *X* or Tyr. During these sequencing analyses, the ^{32}P radioactivity was not eluted with either of the amino acid residues but quantitatively adhered to the sequencing filter. About 70–95% of the radioactivity was recovered on the filter after the sequencing analyses. These results suggest that either the cleaved [^{32}P]ADP-ribose or [^{32}P]ADP-ribosyl amino acid adhered to the filter. Since no amino acid was detected in *X*, it was likely that *X* was an ADP-ribosyl amino acid and was not eluted out from the filter. We therefore screened peptides from similarly digested unmodified G_b for the corresponding sequence. By this we obtained one peptide containing the sequence of Thr-Val-Phe-Glu-Asn-Tyr. Sequence analysis of this peptide is shown in Table II. Comparison of it with that of the [^{32}P]ADP-ribosylated peptide obtained in experiment 3 clearly identified *X* as asparagine. Other amino acid residues including tyrosine showed the comparable recoveries on sequencing of the ADP-ribosylated and unmodified peptides. These results strongly suggest that the asparagine residue is the ADP-ribosylation site of G_b. To corroborate this, we examined the stability of the ADP-ribose G_b bond in 0.5 M hydroxylamine at neutral pH. As shown in Fig. 2, no decrease in the protein-associated radioactivity was found on the at least 5-h incubation, suggesting that this bond was stable under these conditions.

To clarify the functional significance of this ADP-ribosylation, we next examined the effect of the ADP-ribosylation on the basal activities of GTPγS binding and GTPase of G_b. As shown in Fig. 3, neither of the activities was affected by the prior ADP-ribosylation.

DISCUSSION

The present study has clarified the amino acid sequence at the modification site of G_b. The sequence common to all the isolated peptides was *X*-Tyr. Comparison of the sequence of the ADP-ribosylated peptide with that of unmodified one revealed *X* as asparagine, and this amino acid was concluded as the acceptor for the ADP-ribose from several reasons. First, the radioactivity associated with [^{32}P]ADP-ribosylated peptide was not eluted from the sequencing filter, and neither was this asparagine. Tyrosine, on the other hand, showed comparable recoveries in the ADP-ribosylated and unmodified peptides. Second, the linkage between the ADP-ribose and G_b was quite stable in neutral hydroxylamine, which was consistent with the *N*-glycosidic bond involving an asparagine residue. Thus far, no activity catalyzing the transfer of the ADP-ribose to an asparagine has been reported. Thus, the botulinum toxin catalyzes the transfer of the ADP-ribose to the novel amino acid in the novel protein.

It is already known that the ADP-ribosylations of G-proteins affect their functions. For example, ADP-ribosylation of G_i and G_t by pertussis toxin prevents interaction between receptor and G-protein (16, 17). That of G_s by cholera toxin inhibits receptor-stimulated GTPase activity (18, 19). In this study, we found that ADP-ribosylation of G_b by C_1 toxin did not change its basal activities of GTPase and GTPγS binding. This suggests that the ADP-ribosylation does not occur at its GTP-binding site. We have already reported that G_b is probably identical with a putative *rho* gene product (8). When the amino acid sequence of the ADP-ribosylation site was examined against the deduced sequence of the *rho* gene, it was apparent that the ADP-ribosylated asparagine is located in the sequence of Thr^{37}-Val-Phe-Glu-Asn-Tyr^{42}. The *rho* gene is a homologue of the *ras* genes, and this sequence corresponds to Thr^{35}-Tyr^{40} in the *ras* p21 (20). In the *ras* genes, amino acid substitution in this region such as those at positions 35, 36, 38, and 40 has been shown to reduce the biological effect of *ras* p21 without a change in its GTPase- and GTPγS-binding activities, suggesting that this region constitutes the domain that interacts with an effector molecule (21, 22). Recently, GAP (GTPase-activating protein) has been proposed as an effector for *ras* p21 (23), and point mutations at position 35, 36, or 38 amino acid residue of *ras* p21 abolished its interaction with GAP (24, 25). It is not yet known whether GAP for *ras* p21 can interact also with the *rho* gene product or whether there is another GAP-like molecule for this protein. These results, however, raise the possibility that the ADP-ribose acceptor region of this protein also constitutes its effector domain for a GAP-like molecule and the ADP-ribosylation interferes or activates its interaction with such an effector molecule. This possibility will be examined in our future study.

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